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Age-Dependent Effects of Conditioning on Cholinergic and Vasopressin Systems in the Rat Suprachiasmatic Nucleus

Barbara A.M. Biemans^{1,*}, Eddy A. Van der Zee² and Serge Daan¹

¹ Animal Behaviour,

² Molecular Neurobiology, Centre for Behaviour and Neurosciences (CBN), University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands

*Corresponding author

Active shock avoidance was used to explore the impact of behavioural stimulation on the neurochemistry of the suprachiasmatic nucleus. We have found previously that the expression of muscarinic acetylcholine receptors in the suprachiasmatic nucleus of young rats was significantly enhanced 24 hours after fear conditioning. Here, we investigated whether this observation is age-dependent. We used 26 month-old Wistar rats with a deteriorated circadian system, and compared them with young rats (4 months of age) with an intact circadian system. Vasopressin, representing a major output system of the suprachiasmatic nucleus, was studied in addition to muscarinic receptors. Young rats showed a significant increase in immunostaining for muscarinic acetylcholine receptors 24 h after training, corroborating earlier observations. Aged rats did not show such an increase. In contrast, aged rats did show an increase in vasopressin immunoreactivity 24 h after fear conditioning, both at the level of content and cell number, while young rats did not reveal a significant rise. Thus, it seems that these two neurochemical systems in the suprachiasmatic nucleus are regulated independently. The results further demonstrate that the circadian pacemaker is influenced by fear conditioning in an age-dependent manner.

Keywords: Active shock avoidance / Astrocytes / Circadian system / Immunocytochemistry / Muscarinic acetylcholine receptors / Wistar rats.

Introduction

The basal forebrain cholinergic system, acting *via* muscarinic acetylcholine receptors (mAChRs), is critically involved in fear conditioning and learning and memory processes (Lo Conte *et al.*, 1982; Dekker *et al.*, 1991; Van der Zee and Luiten, 1999c). The major function of the cholinergic system is to evaluate sensory stimuli for their

importance (Sarter and Bruno, 1994; Blokland, 1996), and cholinergic neurones play a key role in behavioural arousal and attentive processes (Hasselmo, 1995; Acquas *et al.*, 1996; Wenk, 1997). Cholinergic neurones of the medial septum, nucleus basalis and diagonal band project to the suprachiasmatic nucleus (SCN) (Bina *et al.*, 1993), the master clock of the circadian system in mammals. These cholinergic projections consist of fibres containing large numbers of varicosities, and make axosomatic and axodendritic synaptic contact with SCN neurones (Kiss and Halász, 1996).

The distribution of mAChRs in the rat SCN has been described in detail using the monoclonal antibody M35, which binds to all five mAChR subtypes (Van der Zee *et al.*, 1991; Carsi-Gabrenas *et al.*, 1997). Using subtype-selective antibodies, we reported that SCN neurones express at least m1, but not m2 mAChRs, although both subtypes are present on axon terminals within the SCN (Van der Zee *et al.*, 1999a). In contrast to other brain regions, levels of acetylcholine (ACh) and mAChRs in the SCN do not show endogenous circadian fluctuations in rats or hamsters (Murakami *et al.*, 1984; Van der Zee *et al.*, 1991; Bina *et al.*, 1998). The key function of cholinergic signal transduction in the SCN is currently unclear.

Recently, Van der Zee and co-workers have suggested a new role for SCN-mAChRs in fear conditioning. An increase in the number of mAChR-positive astrocytes following active shock avoidance (ASA) training was seen 2 hours after the last training trial, while an increase in mAChR expression in neurones became apparent approximately 24 h after the last training trial. The novelty aspect of fear conditioning (*i.e.*, becoming familiarised with the test apparatus) was found sufficient to set in motion these SCN-mAChR changes. The investigators hypothesised that these alterations in the SCN may indicate that 'familiarisation with a novel environment in a short and restricted moment in time is placed in a temporal, circadian context' (Van der Zee *et al.*, submitted for publication). By this it is meant that an (unexpected) new situation can receive a 'time stamp' as to mark the circadian phase and allow the organism to anticipate a potential next encounter the following day.

In this follow-up study, we addressed two additional questions. Firstly, does the expression of mAChRs in the SCN of old rats also change in response to fear conditioning (ASA training) as seen in the SCN of young rats? Ageing of the circadian system is accompanied by many changes in behaviour and physiology. In ageing individuals, a damped amplitude is one of the most consistent

findings in overt rhythms, physiological parameters (such as body temperature), hormone levels, and neuronal firing rate (Satinoff *et al.*, 1993; Watanabe *et al.*, 1995; Van Someren *et al.*, 2002; for reviews see Van Gool and Mirmiran, 1986; Weinert, 2000). Other hallmarks of a deteriorated circadian system are the occurrence of more fragmented rhythms (Slonaker, 1912; Davis and Viswanathan, 1998) and changes in synchronisation to external *Zeitgeber* (Scarborough *et al.*, 1997; Duffy *et al.*, 1998). The cholinergic system deteriorates with ageing, underlying cognitive decline in normal ageing and even more so in pathological conditions (e.g. Alzheimer's disease; Bartus *et al.*, 1982; for a review see Muir, 1997). Van

der Zee *et al.* (1991) reported a decrease in mAChRs in SCN neurones in about half of the group of aged rats studied, and an increase in number of mAChR-positive astrocytes in all aged subjects. Taking these many ageing aspects together, familiarisation with a novel environment in a circadian context may be severely impaired in old rats, which may be reflected in SCN neurochemistry.

Secondly, is the vasopressin (AVP) system affected by ASA training? AVP is a neuropeptide abundantly expressed in the SCN and considered a major output system, although its function remains largely unclear. AVP peptide and mRNA expression show endogenous circadian fluctuations (for a review see Van Esseveldt *et al.*,

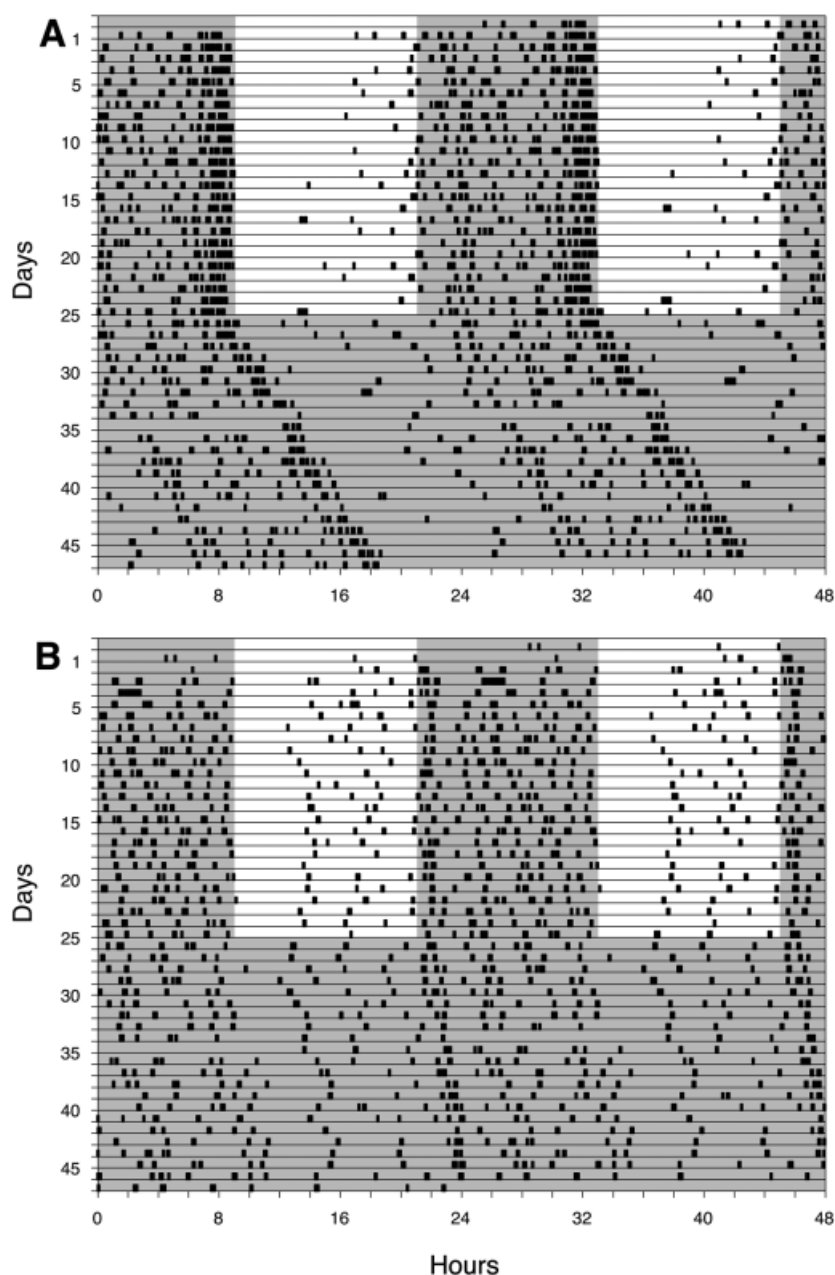


Fig. 1 Double-Plotted Actograms of Representative Examples of Feeding Activity of a Young (A) and an Aged (B) Wistar Rat. Grey areas indicate the dark phase under LD and DD light conditions. NB: light conditions during the dark phase of the LD cycle and the DD period are identical (dim red light).

Table 1 Circadian Feeding Behaviour and ASA Performance.

Circadian Rhythm	Young (n=19)	Aged (n=15)
Amplitude (LD)	711.00 ± 66**	345.00 ± 40
Total activity (LD)	4001.00 ± 353	3094.00 ± 353
τ	24.29 ± 0.03***	24.05 ± 0.04
Δ QP	325.00 ± 25***	91.00 ± 12
SD centre of gravity	1.30 ± 0.12***	2.30 ± 0.26
α	14.00 ± 0.18***	15.90 ± 0.37
ASA performance (%)	Young (n=13)	Aged (n=11)
CAR	30 ± 5**	10 ± 2
CAR last 10 trials	56 ± 8***	9 ± 3
ER 1 st 30 trials	15 ± 2	12 ± 2
ER 2 nd 30 trials	12 ± 3	8 ± 2

Asterisks indicate significant differences between young and aged rats (** $p < 0.01$; *** $p < 0.001$). τ : free-running period; Δ QP: strength of period; SD: standard deviation; α : activity period; CAR: correct avoidance response; ER: escape response.

2000). AVP levels in the SCN have been reported to decrease with ageing in rats (Roozendaal *et al.*, 1987; Biemans *et al.*, 2002), voles (Van der Zee *et al.*, 1999b), and humans (Hofman and Swaab, 1994). AVP release in the paraventricular nucleus of the hypothalamus (PVN), a projection site of the SCN, was shown to be higher in aged rats (Keck *et al.*, 2000). We have found that SCN-AVP may play a role in the circadian modulation of memory retention for fear conditioning in young individuals (Biemans *et al.*, unpublished results). This makes AVP another interesting candidate for a role in circadian modulation of memory processes related to fear conditioning, and possibly as an SCN output system in relation to the alterations seen in mAChR-positive SCN cells.

Results

Circadian Rhythms

The aged rats had a clearly deteriorated circadian system, with more fragmented rhythms and decreased amplitude. Wheel running was hardly observed with aged rats, so this measure could not be used for further behavioural analyses. Representative examples of feeding patterns of one young and one aged rat are given in Figure 1. The rhythm amplitude was different for young and aged rats, whereas total daily feeding was similar. This can be explained by an increased activity period (α) (damped rhythm). Note that food intake itself was not measured, but merely the use of the food hopper. Shorter free-running periods (τ), a lower Δ QP, and lower day-to-day precision were also found in aged rats (Table 1).

Active Shock Avoidance Conditioning

Aged rats were severely impaired in ASA task. They were not able to master the task within the 60 training trials

(Table 1). The percentage of escape responses (ERs) did not differ for young and aged rats during the first or second 30 trials (Table 1). During the second half of the session, the number of ERs decreased for both groups [Wilcoxon Signed Rank (WSR), $p = 0.0033$]. However, for young rats it decreased on account of the rise in number of correct avoidance responses (CARs), whereas this increase was not found in aged animals. It was observed that by the end of the training session, aged rats became tired, and sometimes lay down during the inter-trial interval (ITI), explaining why the number of ERs dropped over time in aged rats. As a consequence of worse acquisition, aged rats received more footshocks than young rats did.

For the young rats, the precision of the circadian system (day-to-day variability of the centre of gravity of activity) correlated negatively with CAR (Pearson correlation: $r = -0.6$, $p = 0.032$), suggesting that a well-organised circadian system is an advantage for ASA learning.

Muscarinic Acetylcholine Receptor Immunoreactivity

In Figure 2, the optical density (OD) of mAChR staining in naïve and ASA-trained animals is depicted. Naïve young and aged rats do not differ in mAChR immunoreactivity (ir). Twenty-four hours after ASA, there is an almost 2-fold increase in mAChR density in young rats [Mann-Whitney U (MWU), $p = 0.007$]. This increase is not seen in the older individuals. For astrocytes in the SCN, the picture is different. Naïve aged rats have ± 2.5 -fold more mAChR-positive astrocytes than young rats (Figure 3, MWU, $p = 0.011$). In the SCN of ASA-trained young rats, the number of astrocytes expressing mAChRs is significantly increased (MWU, $p < 0.001$), but in aged rats no such increase is observed. The contribution of glial mAChR staining to SCN OD is relatively small as can be concluded from Figure 2. However, lower neuronal mAChR in naïve aged rats could be masked to some extent by the mAChR⁺ astrocytes.

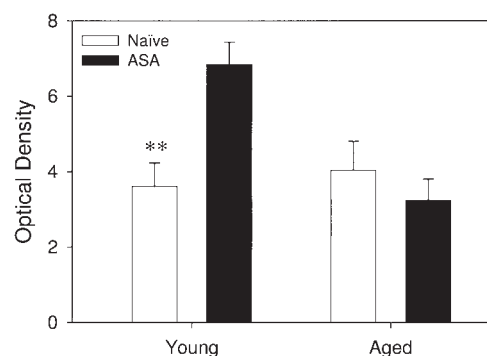


Fig. 2 Optical Density of mAChR Staining in the Middle Portion of the SCN (Level 3) for Young and Aged Naïve Rats (White Bars) and 24 h after ASA Training (Black Bars).

Asterisks indicate significant differences of naïve compared to ASA trained rats. (** $p < 0.01$)

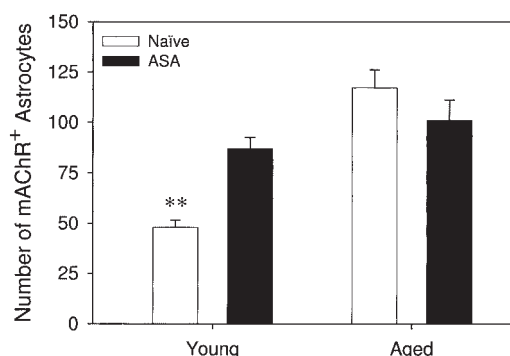


Fig. 3 Cell Counts of mAChR-Positive Astrocytes in the Middle Portion of the SCN of Naïve (White Bars) and ASA Trained (Black Bars) Young and Aged Rats.

Asterisks indicate significant differences between naïve and ASA trained young rats, and between naïve young and aged rats (** $p < 0.01$).

Vasopressin Immunoreactivity

AVP staining intensity was analysed at six SCN levels, and the results are plotted in Figure 4A–C. A rostrocaudal gradient of lower staining at the end levels and higher staining in the middle portion of the SCN can be observed. The statistical analysis was performed over level 2–5, because levels 1 and 6 were not always present for each animal in the available sections. For each individual, ODs of level 2–5 were averaged for statistical evaluation. In addition, AVP⁺ cell counts were performed for SCN levels 3 and 4. The values for these two levels were averaged and are depicted in Figure 4D–F. OD measures correlated strongly with cell counts ($r = 0.65$, $p < 0.001$; one-tailed Pearson correlation).

Young naïve rats had significantly higher AVP-ir than aged rats (Figure 4A; MWU, $p = 0.010$). However, the cell counts (Figure 4D) did not reach statistical significance. ASA training had no significant effect on AVP peptide levels, or AVP containing cell numbers in young rats (Figure 4B, E). In aged rats, ASA had a significant effect on AVP 24 h later. This effect can be observed both on OD (Figure 4C), and the number of AVP-containing cells (Figure 4F). ASA trained aged rats showed a 2-fold increase in OD (MWU, $p = 0.006$), and a 1.5-fold increase in cell counts (MWU, $p = 0.006$).

Discussion

Here we have investigated the effect of fear conditioning on SCN neurochemistry in relation to ageing. We have shown previously that mAChR expression is dramatically increased in the SCN of young rats 24 h after a single session of passive and active shock avoidance training, and that habituation to the test apparatus is sufficient to induce this change (Van der Zee *et al.*, submitted). Our present results confirm the previous findings for young rats; a significant increase in mAChR-ir and the number

of mAChR⁺ astrocytes was found. Apparently, the different fixation techniques used in these two studies (transcardial perfusion vs. immersion fixation here) are of no consequence to this phenomenon.

In order to investigate whether this increase also occurs in individuals with a declined circadian system we extended the study to aged rats. The aged rats had a clearly deteriorated circadian system, based on the observed behavioural changes (decreased amplitude; shorter free-running period and less robust rhythm; longer α ; higher day-to-day variability). All these features of the circadian system were reported to be affected by age (Van Gool and Mirmiran, 1986; Weinert, 2000), and differed significantly for our young and aged Wistar rats. There was no increase in mAChR-ir, or in the number of mAChR⁺ astrocytes, following ASA in the old SCN. The cholinergic SCN cells do not respond to ASA, which suggests that the SCN has become insensitive to this type of stimulation. A reduction in SCN ACh-innervation

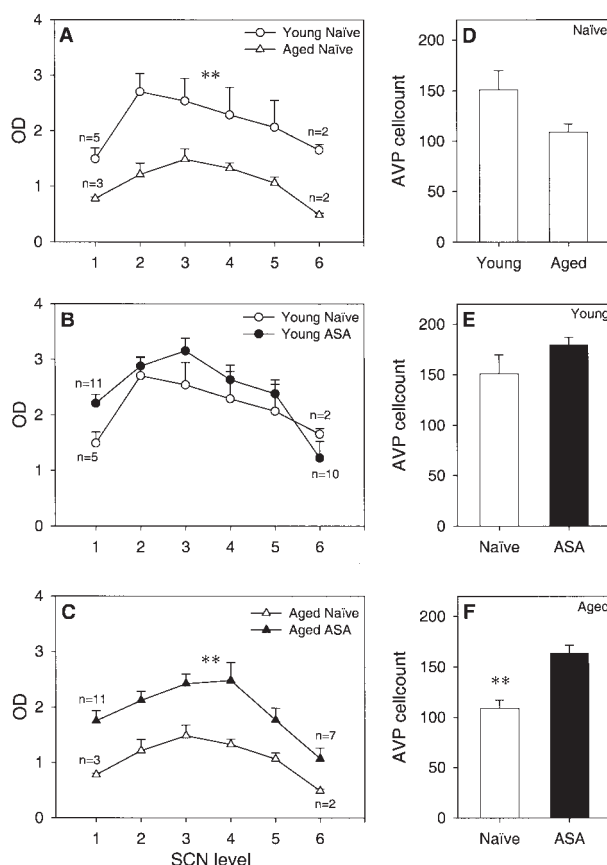


Fig. 4 OD of AVP-ir in the SCN of Young and Aged Naïve (A) and Naïve and ASA Trained Young (B) and Aged (C) Rats at 6 Rostrocaudal Levels.

Numbers of animals per group at the different levels are: young naïve: $n = 6$; aged naïve: $n = 4$; young ASA: $n = 13$; aged ASA: $n = 11$, unless specified otherwise in the graph. Asterisks indicate significant differences between the OD levels 2–5 (** $p < 0.01$). (D–F) AVP⁺ cell counts in the middle portion of the SCN (level 3 and 4) for naïve (D) and naïve versus ASA trained young (E) and aged (F) rats.

due to the well-known decline in the basal forebrain cholinergic system at high age (Muir, 1997) is a possible cause for this.

The parallel increase of astrocytes and mAChR-ir in young rats and the absence of both in old rats suggest a functional link between mAChR⁺ neurones and astrocytes. The number of mAChR⁺ astrocytes is already high in aged individuals, and the lack of increase here could also be due to reaching a plateau level. If astrocytes are a prerequisite for the neuronal response, this could explain the absence of a raise in mAChR OD in aged rats.

In addition to the cholinergic system, we have looked at the vasopressin system, a major output system of the SCN, in response to ASA [one training session and one (reinforced) retention session of 15 trials]. The distribution of AVP staining in the Wistar rat SCN along the rostro-caudal axis was similar to that reported for other strains of rats (Wollnik and Bihler, 1996), mice (Bult *et al.*, 1992), and voles (Gerkema *et al.*, 1994). In all these studies, the SCN was also divided in 6 levels, with high values at levels 3 and 4.

The young rat SCN did not show any changes in AVP OD or AVP cell numbers in response to ASA, except at the most rostral level where AVP OD was enhanced (single MWU test, $p=0.02$). In aged rats, however, there was a clear increase in AVP OD at all SCN levels. In addition, the AVP⁺ cell counts at levels 3 and 4 differed significantly. It is worth noting that the quantification methods used here (OD measurements and cell counts) are different, but complementary. In the cell-counting method, densely stained but also moderately to weakly stained cells are equally counted. This gives an estimate of the number of cells containing AVP peptide. In the computerised quantification (OD) method, cells with low peptide content have lower OD values compared to cells with high peptide content. On the other hand, fibres, as well as varicosities and terminals, contribute to the OD value. Therefore, this latter method gives a (relative) measure of peptide content. The strong correlation between these two parameters, and the fact that both OD and cell counts differ significantly in ASA trained aged rats compared to naïve ones, strengthen the point that these measures are equally well suited for studying neurochemical changes, although they have distinct properties.

It is difficult to interpret the finding of enhanced AVP-ir in aged, but not young rats. High AVP-ir levels in neurones can reflect low AVP release from those neurones, and hence an AVP accumulation inside the neurones. On the other hand, more AVP could be produced as a result of the ASA experience. If this production is induced by stress, it is tempting to speculate that aged rats have an increased production due to the exposure to more footshocks, or the additional stress of no longer being able to respond with ERs anymore due to exhaustion. We cannot decide from this experiment whether the increase in AVP is a 24 h effect due to the training, a result of the direct stress caused by the preceding retention session, or

both. However, it is hard to explain that AVP could be increased as a direct result, because this would imply acute production, and this is unlikely to be accomplished at such short intervals. Alternatively, it would mean a sudden blocked release in case of the young rats, which is also not very obvious. To answer this question, one would have to determine what happens with SCN-AVP directly after the first training session.

Acute effects of stress on the SCN-AVP system have been reported. CSF-AVP levels, most likely originating from the SCN, are enhanced during passive avoidance (Laczi *et al.*, 1984) and swimming stress triggered AVP release within the SCN in rats (Engelmann *et al.*, 1998). Recently, we have observed a stress-induced decrease of AVP-ir in the SCN of house mice (Biemans *et al.*, unpublished results). The SCN projects to, and possibly regulates neurones of the PVN directly as implied by electrophysiological data, or indirectly through vasopressinergic stimulation of GABA-ergic neurones (Hermes *et al.*, 2000). Keck *et al.* (2000) found that aged rats have a 2-fold higher basal release of AVP within the PVN. The intra-PVN rise in AVP release in stressed young rats is blunted in aged individuals. This could possibly be explained by an increase in inhibitory actions through enhanced AVP levels in the aged SCN. The dorsomedial hypothalamus (DMH), another nucleus densely innervated by the SCN, is however not affected by acute stress (Engelmann *et al.*, 1998). These findings all suggest that the circadian pacemaker may be involved in the sequence of adaptive events in physiology and behaviour resulting from a challenge.

From our previous study it appeared that habituation to the ASA apparatus is sufficient to enhance mAChR density significantly. Nevertheless, training and retention caused an additional (non-significant) increase. Here, we exposed the aged rats to the strongest stimulus, and found no effect on mAChR-ir. The increase in mAChRs is not likely to be caused directly by the retention session, since we found in our previous study that changes are not observed in young rats until 22 h after training.

The fact that aged rats were unable to accomplish the task was not due to hearing impairment; hearing was checked for each rat a few days before the experiment by a finger snap made outside its cage. In addition, quite a few aged and young rats were so startled by the first conditioned stimulus (CS, *i.e.* the tone), that they made a spontaneous CAR. When this was not the case, it was made sure by behavioural observation of alertness that the CS was heard. Aged rats were also not less capable to move from one compartment to the other due to motor disabilities; they were well able to make escapes during presentation of the footshock. Finally, although differences in shock sensitivity cannot be excluded, aged rats displayed aversive reactions (paw withdrawal, jumping, vocalisations) to the same degree as young rats did, and thus cannot explain the complete absence of learning.

Conclusions

The increase in mAChRs in young but not aged rats on the one hand, and the AVP enhancement in aged but not young on the other, demonstrate that SCN systems are affected by fear conditioning, but seem to respond independently. Alternatively, increased AVP production might occur in young as well as in aged rats. In young rats this peptide may already have been released, whereas in aged rats it then remains stuck or clogged-up inside the neurones, possibly reflecting a neuronal release deficiency.

Taken together, the SCN cholinergic and vasopressinergic system appear to be age-dependently affected by fear conditioning. Presently we do not know which pathways initiate these changes, and to what extent mAChR⁺ neurones and astrocytes interact; this awaits further investigation.

Materials and methods

Subjects and Housing

Male Wistar rats were used, aged 4 months ($n=19$) or 27 months ($n=16$) at the time of the experiment. Both young and aged rats were purchased from Harlan (Horst, The Netherlands). Aged rats were group housed in large cages (80×40×30 cm; 7–8 animals/cage) from the time of purchase (6 months of age) until onset of the experiment. They were kept on a natural light-dark (LD) cycle, with at least 12 hours of light per day. Young rats were obtained 1.5 years later.

Two months before the experiment, they were moved to light- and temperature controlled ($21\pm2^\circ\text{C}$) climate rooms on a 12:12 h LD cycle with lights on from 10:00 (young and old rats equally divided over the climate rooms). Rats were individually housed in large cages (30×45×50 cm) equipped with a running wheel.

Circadian activity patterns could be monitored by means of running wheel and food hopper recordings. Activity pulses were relayed to a PC-based event recording system and stored in 2-minute bins. Activity was monitored for 7 weeks, starting with a 4-week LD period of 12:12 hours (± 30 Lux), followed by a 3-week free-running period in constant dim red light (DD) (± 1 Lux). After this free-running period, rats were entrained again for 6 days before onset of the experiment. During this period, rats were handled regularly by taking them out of the cage and putting them on the experimenter's lap.

Active Shock Avoidance Apparatus

A fully automated shuttle box (Coulbourn Inst. L.L.C., Allentown, USA) was used for two-way active shock avoidance (ASA). The shuttle box consists of two identical compartments (25×25×30 cm), separated by a clear Perspex wall, with a large opening in the middle. The grid floor is made of stainless steel (50 mm, spaced 130 mm apart) bars through which a scrambled footshock can be delivered. Infrared emitters and receivers detect a rat changing compartments. A 4.5 kHz tone served as the conditioned stimulus (CS), and was presented 5 s prior to the onset of a mild footshock [unconditioned stimulus (US); 0.3 mA for 3 s]. After 8 s, both CS and US were terminated if the rat failed to make a response. If the rat jumped to the other compartment during sole presentation of the CS, a correct avoidance re-

sponse (CAR) was recorded. If the rat jumped to the other side during presentation of US + CS, an escape response (ER) was recorded. The inter-trial interval (ITI) was 20 s. The apparatus was placed in a sound attenuated room, dimly lit by a 15-Watt light bulb.

Experimental Procedure

All experiments were carried out with the approval and under license of the Groningen Animal Experiment Committee. Experimental rats (young $n=13$; aged: $n=11$) were transported in their home cage from the climate room to the experimental room, and placed in the shuttle box with their heads facing away from the opening. They received one training session of 60 trials, between 11:00–18:15. Exactly 24 hours after their individual training time, rats received a (reinforced) retention session of 15 trials of which the procedure was identical to training. Immediately afterwards, they were sacrificed using CO₂ (within the 20-min time slot of the training session on the previous day). Naïve rats (young $n=6$; aged: $n=5$) served as controls, and were sacrificed together with the experimental animals.

Tissue Preparation and Immunocytochemistry

Brains were quickly removed from the skull and immersed in 2.5% paraformaldehyde for 8 hours. Thereafter they were stored in phosphate buffered saline (PBS; pH 7.4) with 0.1% sodium azide to prevent bacterial growth. Twenty-four hours before sectioning, they were put on a 30% sucrose solution at 4°C, for dehydration and cryoprotection. Sections (20 µm) were cut coronally at the level of the hypothalamus on a microtome. After sectioning, free-floating sections were thoroughly rinsed in PBS and pre-incubated for 15 min in 0.1% (for mAChR) or 0.3% (for AVP) H₂O₂ in PBS, and immersed in 5% normal rabbit serum (NRS) in PBS for 30 min (in case of mAChR) to reduce non-specific binding in the following incubation step. Then the sections were incubated with the first antibody, the monoclonal mouse anti-mAChR IgM M35, raised against purified bovine mAChR-protein as described in detail elsewhere (Van der Zee *et al.*, 1989) or the monoclonal mouse-anti-AVP 1:500 (PS41, kindly supplied by Dr. H. Gainer, NIH, USA).

M35 was diluted 1:5 and PS41 1:500 in PBS, [with 1% normal sheep serum (NSS) added for PS41], and incubation was done overnight at room temperature (RT), under gentle movement. M35 does not discriminate between the five mAChR subtypes (Carsi-Gabrenas *et al.*, 1997; Van der Zee and Luiten, 1999c). After the primary incubation, sections were rinsed in PBS and again pre-incubated with 5% NRS in PBS for 30 min (M35) before the secondary incubation step (1.5 h at RT) in biotinylated rabbit IgG anti-mouse-IgM [μ -chain directed, F(ab') fraction, Zymed], diluted 1:200 in PBS for M35 or in biotinylated sheep-anti-mouse IgG (Amersham) diluted 1:800 in PBS for AVP. Subsequently, sections were thoroughly rinsed in PBS and incubated in streptavidin-HRP (Zymed), diluted 1:200 (mAChRs) or 1:500 (AVP) in PBS for 2 h at RT. For AVP, Triton X-100 (0.5%) was added to all incubation steps. Finally, after rinsing in PBS, the sections were processed by the diaminobenzidine (DAB)-H₂O₂ reaction (0.03% DAB and 0.002% H₂O₂ in PBS), guided by a visual check. Control experiments were performed by the omission of the primary antibody, yielding immuno-negative results.

Data Analysis and Quantification

Circadian rhythms Activity records of 16 days of LD and 10 days in free-running conditions were analysed in order to characterise the circadian system. The amplitude of circadian

rhythms (*i.e.* highest peak) and average total daily feeding activity was determined in LD. In DD, τ (free-running period) and the robustness of the circadian system was determined by means of χ -square periodogram analysis (Sokolove and Bushell, 1978). The periodogram was calculated on qualitative data (activity in 2-min intervals scored as 1; absence of activity as 0), and provides significant frequencies and their relative strength (ΔQP). Furthermore, the inter-daily variation of the centres of gravity (highest intensity of activity) was calculated to provide a measure of precision, and the range of the activity period (α) was calculated by subtracting time of activity offset from time of onset.

Immunocytochemistry One rat of the aged control group was excluded from further analyses due to a large tumour inside the third ventricle, which severely damaged the SCN. The optical density (OD) of mAChR immunoreactivity (mAChR-ir) and AVP-ir was determined in the SCN. The SCN was divided into 6 rostrocaudal levels, with levels 1 and 6 corresponding to bregma levels -0.92 and -1.6 mm, respectively, according to the brain atlas of Paxinos and Watson (1998). From earlier studies, it appeared that mAChR immunostaining after fear conditioning was most dramatically increased at level 3 (Van der Zee *et al.*, submitted). Therefore, the OD for mAChRs was determined at level 3, whereas that for AVP was determined at levels 1–6. Left and right SCN measurements were averaged. The OD was expressed in arbitrary units corresponding to grey levels using a Quantimet 600 image analysis system (Leica). Background staining was measured in the vicinity of the SCN, devoid of specific staining. The relative OD was calculated by the equation $[(OD_{\text{area}} - OD_{\text{background}})/OD_{\text{background}}]$, thus correcting for between-section (background) staining variability.

For AVP, the number of immunopositive cells was also counted at levels 3 and 4, to have an additional measure of AVP immunostaining. For mAChRs, the number of immunopositive astrocytes was counted at level 3.

Statistical Evaluation Non-parametric Mann-Whitney U tests (MWU) or Wilcoxon Signed Rank (WSR) tests were used where appropriate. Results are displayed as the average \pm the standard error of the mean (SEM). Pearson correlations were used to investigate relationships between parameters. In all cases, a p -value of <0.05 was taken as an indicator of significance. All tests were applied two-tailed unless specified otherwise.

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